N-acetyl aspartate (NAA) is a marker of disability in secondary progressive multiple sclerosis

N-acetyl aspartate (NAA) is a marker of disability in secondary progressive multiple sclerosis: a proton magnetic resonance spectroscopic imaging study

Abstract

Background and Purpose:

We undertook a ^{1}H-MRS imaging study in a large secondary progressive MS (SPMS) cohort, to examine whether metabolic markers of brain injury are associated with measures of disability.

Methods:

A cross-sectional analysis of people with SPMS was performed in 119 participants. They underwent ^{1}H-MRS to obtain estimated concentrations and ratios to total Cr for total NAA, mIns, Glx and total Cho in normal appearing WM (NAWM) and GM. Clinical outcome measures chosen were: Paced auditory serial addition test (PASAT3), Symbol digit modalities test, nine-hole peg test (9HPT), Timed 25-foot walk and Expanded Disability Status Scale. The relationship between these neurometabolites and clinical disability measures was initially examined using Spearman rank correlations. Significant associations were then further analysed in multiple regression models adjusting for age, gender, disease duration, T2 lesion load, normalised brain volume and occurrence of relapses in two years preceding study entry.

Results:

Significant associations which were then confirmed by multiple linear regression were found in NAWM for: tNAA/tCr and 9HPT [Rho=0.23, 95% CI [0.06-0.40]; tNAA and tNAA/tCr and PASAT3 [Rho=0.21, 95% CI [0.03-0.38], [Rho=0.19, 95% CI [0.01-0.36]; mIns/tCr and PASAT3 [Rho= -0.23, 95% CI [-0.39 -to -0.05]; and in GM for tCho and PASAT3 [Rho=-0.24, 95% CI [-0.40 to -0.06]. No other GM or NAWM relationships were found with any

metabolite, with associations found during initial correlation testing losing significance after multiple linear regression analysis.

Conclusion:

This study suggests that metabolic markers of neuroaxonal integrity and astrogliosis in NAWM, and membrane turnover in GM may act as markers of disability in SPMS.

Abbreviation Key	
9HPT	Nine-hole peg test
CSI	Chemical shift imaging
EDSS	Expanded disability status scale
IPS	Information processing speed
NAWM	Normal appearing white matter
PASAT3	Paced auditory serial addition task (3 second)
SDMT	Symbol digit modalities test
SPMS	Secondary progressive multiple sclerosis
T25FW	Timed 25-foot walk
WML	White matter lesion

INTRODUCTION

Secondary progressive multiple sclerosis (SPMS) is the dominant progressive form of multiple sclerosis that is characterised by accumulating disability due to a variety of neurodegenerative processes.[1] These include microglial activation with subsequent formation of reactive oxygen species inducing mitochondrial damage; sodium channel dysfunction leading to histotoxic hypoxia and axonal energy failure; and glutaminergic excitotoxicity.[2–4]

Surrogate markers of brain injury are valuable in improving our understanding of the pathophysiology driving clinical disability in progressive MS. Surrogate imaging-based markers such as MRI based lesional and atrophy metrics, can identify existing inflammatory injury and axonal loss and provide adjunctive prognostic information. Yet existing imaging based-measures are relatively limited in their ability to demonstrate metabolic or microstructural changes and show only a modest association with clinical disability outcomes

in progressive MS (PMS).[5] This is where advanced non-structural MRI techniques such as ^{1}H-MRS are attractive to further understand this neuropathology and its association with clinical disability in progressive forms of MS.

Using ^{1}H-MRS, neurometabolites of interest in MS include: N-acetyl aspartate plus N-acetyl aspartyl glutamate (total NAA = tNAA), a marker of neuroaxonal integrity and mitochondrial function;[6,7] Glx – the sum of the excitatory neurotransmitter glutamate and its precursor glutamine;[4] myo-inositol (mIns), a marker of glial cell activity, most likely astrogliosis; and total Choline (tCho = glycerophosphocholine and phosphocholine) – a marker of membrane turnover.[7,8] Many studies have demonstrated decreases in tNAA and tNAA/tCr; and increases in total creatine (tCr = creatine and phosphocreatine) and inositol in normal appearing white matter (NAWM) and GM in SPMS.[9] In a recent meta-analysis of ^{1}H-MRS studies, effect sizes for a reduction in NAA and NAA/Cr were larger in PMS compared to relapsing-remitting MS.[9] There have been conflicting results from studies examining disability associations in PMS: several studies showed no association between metabolites (NAA, Glx, mIns, tCho) and the Expanded disability status scale (EDSS);[10–13] whilst others showed moderate associations with EDSS, nine-hole peg test (9HPT), Timed 25 foot walk test (T25FW) in cortical GM and NAWM.[14–16] Of the studies examining cognitive performance [including information processing speed (IPS)] in PMS, no associations were found in sample sizes ranging from 14-31, with only two of these studies containing pure SPMS cohorts.[13,14,16–18]

The rationale for this cross-sectional study was to further define metabolite levels, and their associations with disability, in a much larger sample of people with SPMS than has been achieved before.

Methods:

Participants and measures

Participants were recruited from the MS- Secondary Progressive Multi-Arm Randomisation Trial (MS-SMART) (NCT01910259) – a phase 2b double-blind, placebo-controlled, multi-arm, multi-centre study assessing the neuroprotective potential of amiloride, fluoxetine and riluzole in SPMS. Details of the trial protocol and final trial results were recently published.[19,20] Participants recruited into the study were people with SPMS aged 25-65 with an EDSS score of 4.0-6.5, that showed evidence of progression independent of relapses over the last 2 years. Participants were randomised 1:1:1:1 to amiloride, fluoxetine, riluzole or placebo, and the primary outcome measure was the percentage brain volume change over 96 weeks.

Participants involved in the MS-SMART study at our site (Queen Square MS Centre, University College London) were invited to take part in an optional "Advanced MRI sub-study" inclusive of ^{1}H-MRS scans. Recruited participants had their MRI scan and clinical assessments prior to randomisation and commencing study medication. They underwent a series of clinical assessments including EDSS, T25FW, 9HPT and two standard measures of IPS: Paced Auditory Serial Addition Test-3 (PASAT3) and Symbol Digit Modalities Test (SDMT).[21–23] MRI measures included normalised brain volume, normal appearing WM (NAWM), cortical GM and T2 lesion volume.

Consent was obtained for all participants according to the Declaration of Helsinki and ethical approval for the study was provided by the Scotland A Research Ethics Committee [13/SS/0007].

MRI acquisition

Neurometabolite spectra from multiple voxels within one scan were acquired using a technique known as chemical shift imaging (CSI) to determine estimated neurometabolite concentrations and ratios by obtaining average metabolite concentrations from a slice of neuronal tissue.[24]

Imaging was acquired using a Philips Achieva 3T MRI scanner (Philips Healthcare, Best) using a 16ch neurovascular coil.

All participants underwent the following scans:

MRS: A 210x160mm^2 VOI with 15mm slice thickness was selected for CSI, placed superior to the lateral ventricles (Fig. 1). The inferior margin of the slice was positioned at the superior margin of the corpus callosum, angulated to the anterior commissure/ posterior commissure line. At the baseline visit, a screen shot of the exact positioning of the CSI was saved (see Fig. 1) to be used as a reference for subsequent time points. The slice placement was chosen to avoid the ventricles to ensure that all voxels were of consistent quality and shim. The CSI VOI was subdivided into a 21x16 grid giving a voxel size of 10x10x15mm^3. Spectra were acquired using the manufacturer's 2D Point RESolved Spectroscopy sequence (short TE 35ms/ TR 2000ms). Outer volume suppression using fat saturation was applied to limit artefacts, and the VOI was shimmed using the pencil beam-auto technique.[25] CHEmical Shift Selective saturation pulses were used for water suppression. A reference scan with no water suppression was also collected with identical parameters during the same exam for quantification. tNAA (total NAA = N-acetyl aspartate and N-acetyl aspartyl glutamate), mIns, Glx (sum of glutamate and glutamine), tCho (total choline = glycerophosphocholine and phosphocholine) and each of their ratios to total creatine (sum of creatine and phosphocreatine) were calculated.

Structural MR imaging: Scans for structural information and lesion assessment were collected prior to MRS as detailed below and were used also for planning purposes. A sagittal 3D-T1WI with matrix 256 x 256, FOV 256 x 256mm^2, 180 sagittal slices 1mm thick, flip angle 8^o, TR/TI/TE = 7/840/3.2 ms (turbo factor 230) was acquired for structural information. An axial PD-T2 (TR/TE1/TE2) = 3500/19/85 ms, turbo factor 10) and FLAIR (TR/TI/TE =

8000/2400/125 ms, turbo factor 24) with matrix 240 x 180, FOV 240 x 180 mm^2, 50 slices 3mm thick were acquired for lesion assessment.

MRI analysis

Brain and WM lesion segmentation

In order to obtain segmentations of WM, GM and CSF, a NIFTI image was created in the same space as the axial PD-T2 to represent the positioning of the CSI matrix and to act as a mask for the MRS volume (Fig. 1). The axial PD-T2 image was rigidly registered and resampled to the 3D T1 image space using NiftyReg,[26] and an identical transform applied to the CSI mask to get the matrix in the desired space for segmentation. WM lesions were semi-manually delineated using Jim 7 (Xinapse Systems) on the T2WI using the FLAIR image as a reference. After lesion filling,[27] the 3D-T1WI was segmented using geodesical information flows enabling the calculation of T2 lesion volume.[28]

Brain volume measures

Normalised brain volume was calculated using the SIENAX method from the segmented 3D-T1WI.[29] SIENAX rescales each subject head to an Montreal Neurological Institute atlas size, hence correcting each individual brain volume. Thus, the normalised brain volume is the volume of the rescaled brain and it allows us to correct brain volume for variations in head size, effectively resulting in a measure of cross-sectional atrophy.[29]

CSI Spectra & image post-processing:

Following the acquisition, post-processing of spectra was completed using Linear Combination of Model Spectra (LCModel© version 6.3-1A) – a program used to fit MRS data to a basis set, which together with the water reference scan, enabled estimated quantification of metabolites

as well as providing a set of parameters to perform quality assurance for each voxel.[30] The LCModel basis set was provided by S. Provencher (personal communication).

The water reference scan was used to find a scaling factor for the basis set, as detailed in the LCmodel manual.[30] This carries with it the assumption that the concentration of water in the spectrum is equivalent to healthy white matter (35880mM), which, in the absence of a specific measure per patient is often used as the default.[30,31] In addition to this to correct for T2 relaxation the default water attenuation was set to 0.7, based on TE=30 and on Ernst et al. who found the major water compartment in brain has T2 ≈ 80 ms. (exp(−TE/T2) = exp (−30/80) ≈ 0.7).[30,31] If substituted for TE=35ms this reduces to 0.65, however in the absence of an accurate T2 in pathology, the default was again kept (Sec 11.2 LCmodel manual). As we employed long TR and water scaling was used, no correction for T1 was made.

Spectra from individual voxels were automatically rejected if any neurometabolite Cramer-Rao lower bounds were > 20%, full width half-maximum of the tNAA spectral peak was >15Hz or SNR < 9. All voxels that passed the automated step were visually inspected by an experienced assessor (BS) to look for baseline artefacts, non-random residuals or outer volume contamination that may prevent the accurate measurement of neurometabolites. The resulting set of voxels made up a "clean data" set which all passed the automated and visual QA.

Figure 2. An example spectrum from a single voxel of brain tissue and spectra from the chemical shift imaging grid

Neurometabolite analysis:

Neurometabolites were analysed in the following two ways:

i. **Estimated metabolite concentrations:** The mean metabolite concentrations (tNAA, mIns, Glx, tCho) of all voxels which passed the automatic and visual quality assurance

(clean data) were averaged for each participant to calculate per patient estimated metabolite concentration reported in institutional units (i.u.).

ii. **Metabolite ratios**: Mean neurometabolite ratios (tNAA, mIns, Glx, tCho) to total creatine (tCr) were calculated from the clean data for each participant.

Metabolite concentrations and ratios were then calculated in the following two tissue types:

i) Normal appearing white matter (NAWM)

ii) Grey matter (GM)

To eliminate contamination by WML and CSF, we firstly removed any voxels containing > 1% WML and > 15% CSF. Linear regression models were used to explore the association between the concentration of each metabolite and white or grey matter fraction on a voxel by voxel basis. These models were then extrapolated to find the value for each metabolite where GM or WM fraction was 100% to find the metabolite concentration for each tissue type. Metabolite outliers were removed from the regression models leaving us with per patient metabolite values. This method has been previously described.[32] Owing to the small lesion load in the acquired slice (average WML fraction in SPMS cohort was 0.01), calculation of metabolite values in WML was unable to be completed.

Statistical analyses

Neurometabolic data and distribution over the cohort was summarised descriptively. Statistical analysis was completed using R statistical software (version 3.5.1).[33] All p values were two tailed and reported using a 0.05 significance level.

Neurometabolite correlations with clinical disability measures:

To examine the association between tissue specific neurometabolites and clinical disability measures (EDSS, T25FW, 9HPT, PASAT3, SDMT), Spearman rank correlation coefficients

were calculated given the non-normal distribution of the clinical disability measures. The scope of the analysis was to analyse the association between our key metabolites in NAWM and GM (tNAA, mIns, Glx, tCho and their ratios to tCr) and the disability measures listed above. During correlation testing, sixty comparisons were pre-specified but here we report eighty after tCho (and tCho/tCr) were added following a post-hoc request by a peer reviewer. These are reported in Table 3. In reporting multiple analyses, we were guided by considerations outlined in Patel C et al.: multiplicity adjustment is not required when a list of hypotheses of primary importance are pre-specified; emphasis is placed on being explicit and transparent about the extent of multiplicity; and the magnitude of observed associations is interpreted in the context of the background literature.[34]

Where statistically significant correlations were found in the Spearman rank correlation analysis, a subsequent multivariable linear regression analysis was performed, adjusting for age, gender, disease-duration from onset, occurrence of relapses in the preceding two years, T2 lesion volume and normalised brain volume. Model diagnostics undertaken to assess the regression model included: calculation of variance inflation factors to determine multi-collinearity; Cook's distance to determine effect of leveraged data points; plots of studentised residuals against adjusted predicted values to check residual homoscedasticity.

Results:

154 participants gave consent for the Advanced MRI sub-study from January 2015 to June 2016. Of these 154 participants, six failed the CSI quality assurance leaving 148 participants in the CSI arm of the Advanced MRI sub-study at baseline.

In total there were 8361 CSI voxels for the analysis which passed both automatic and visual quality assurance. 3035 voxels remained after excluding voxels that contained > 1% WML and > 15% CSF (mean 20.5 voxels per patient, standard deviation 9.2). After calculating metabolite

values and removing metabolic outliers, 119 SPMS participants remained for analysis. Cohort demographics are provided for the cohort of 119 participants that underwent correlation testing and regression analysis. 81/119 (68%) were female and 108/119 (91%) had not experienced a relapse in the two years before randomisation. Further details of the cohort are shown in table 1.

Table 1. Cohort demographics and characteristics

Estimated neurometabolite concentrations (in i.u.) and metabolite ratios to tCr can be found in Table 2.

Table 2. Summary statistics for neurometabolite estimated concentrations (i.u) and ratio to total creatine

Metabolite concentrations/ratios which showed statistically significant correlations to disability measures in NAWM and GM are shown in Table 3.

Table 3. Correlations between neurometabolites and clinical disability measures

Table 4 shows the results of the regression analysis for neurometabolites that were significantly associated with clinical disability measures after adjusting for covariates. Neurometabolites that showed a statistically significant association on the Spearman rank correlation but lost statistical significance after regression analysis are not shown in table 4. In NAWM,

associations were seen between tNAA/tCr and 9HPT (β = 0.23, p = 0.04); tNAA and tNAA/tCr and PASAT3 (β = 0.17, p = 0.04), (β = 0.19, p = 0.02) respectively; and mIns/tCr and PASAT3 (β = -0.22, p = 0.007). In GM, tCho was associated with PASAT3 (β = -0.27, p = 0.04).

Table 4. Statistically significant associations between neurometabolites and clinical disability measures from regression analysis

Figure 3. Associations between neurometabolites and clinical disability measures

Discussion:

This is the largest reported cohort of people with SPMS undergoing ^{1}H-MRS. Table 3 shows all of the correlations with those in bold as statistically significant. They were then explored in a multiple regression analysis (table 4). The results suggest a relationship in NAWM between tNAA (tNAA/tCr) and mIns/tCr and IPS performance (PASAT3); and between tNAA/tCr and upper limb function (9HPT). In GM, tCho was associated with IPS performance. Whilst a number of other correlations were identified in the correlation analysis, no associations with EDSS or T25FW were found in GM or NAWM after multiple regression analysis.

Relationships between neurometabolites and clinical disability (refer table 4):

Upper limb function

Previous smaller studies examining tNAA or tNAA/tCr in NAWM in PMS have not demonstrated statistically significant associations between neuroaxonal integrity and upper limb function.[14,16] Our study suggests that as neuroaxonal integrity (and mitochondrial function) decreases, upper limb function, as reflected by 9HPT, also decreases. The association found between tNAA/tCr and 9HPT during the correlation analysis (see table 3) and multiple

regression head in the expected direction with decreased tNAA/tCr associated with decreased upper limb function (as reflected by 9HPT). It remains of interest whether this is reflective of regional changes in NAWM that affect specific tracts related to upper limb and hand function or are the changes more reflective of generalised changes throughout the NAWM. With the association being seen with 9HPT but not T25FW, it may suggest that changes in neuroaxonal integrity and mitochondrial function in the brain play a more significant role in upper limb dysfunction in the progressive stage of the disease. This could be further explained by the hypothesis that PMS is a length dependent central axonopathy whereby legs are affected earlier due to greater susceptibility of spinal cord motor neurons; and the greater reserve capacity of shorter neuronal pathways such as the upper limb earlier in progressive disease.[35] Sastre-Garriga et al found an association between tNAA and 9HPT in cortical GM ($r = -0.48$, $p = 0.03$) and in this context the correlation in this study of 0.22 (see table 3) is lower – although our association was in NAWM rather than cortical GM.[14] Our result contrasts with previous studies but this could be explained by the differences in cohort characteristics whereby Sastre-Garriga et al examined 43 predominantly male participants with early PPMS with a lower median EDSS of 4.5 and Obert et al analysed 15 SPMS with a median EDSS 4.5.[14,16]

Information processing speed:

A previous much smaller study examining measures of IPS (PASAT3) and its relationship to in vivo neurometabolites in NAWM in SPMS ($n = 15$) did not show any associations.[16] Several other studies reported results in the form of standardised scores such as MS Functional composite or the Brief Repeatable battery, making it difficult to discern the true relationship between IPS and neurometabolites in PMS.[13,14,18] Firstly, our findings of an association between NAWM tNAA and tNAA/tCr with PASAT3 scores (see table 3 & 4, figure 3) are in the expected direction, with decreased neuroaxonal integrity in NAWM, associated with decreased performance on the PASAT3. We are unable to determine whether there was a

predilection to a specific region of NAWM or whether it was possibly associated with altered functional or structural connectivity. Solana et al examined this in a mixed cohort of relapsing remitting MS and SPMS, demonstrating that NAA/Cr and mIns/Cr in WM were associated with abnormal efficiency in the front-parietal network with abnormalities in this network associated with impaired attention and processing speed (compared to healthy controls).[32] Our findings differed from Obert et al who employed a similar ^{1}H-MRS acquisition to calculate NAA/Cr, but their SPMS cohort of 14 undergoing ^{1}H-MRS may not have had sufficient power to detect an association. Gender is also associated with IPS with females being associated with decreased PASAT3 scores compared to males adjusting for the other covariates (see Table 4). Neurometabolic differences between males and females in SPMS was examined by De Stefano et al. who did not find differences in NAA/Cr in NAWM.[36] There was also no difference in tNAA or tNAA/tCr in NAWM between males and females in our SPMS cohort (welch t test: $p = 0.3$, $p = 0.7$ respectively). Penny et al examined the longitudinal association between NAA and IPS in PMS and did not identify gender as being associated with 5-year measures of IPS. Their results however may differ as they studied a cohort of PPMS that had a predominance of males and a lower baseline median EDSS of 4.5.[37] T2 lesion volume reflecting the inflammatory lesion burden was also associated with IPS and cognitive performance– findings that have been demonstrated before confirming the strong association between inflammatory lesion burden and clinical disability in PMS.[18,37]

We also found that mIns/tCr in NAWM had a negative association with PASAT3 scores (see Table 3 & 4). This relationship is in the expected direction with increased astrogliosis (as reflected by higher mIns/tCr) associated with decreased performance on PASAT3. mIns values were not associated with tests of IPS but the ratio between the two showed a weak negative association which suggests that as mIns/tCr ratio increases, PASAT3 scores decrease (see figure 3). This finding could be driven by either an increased mIns or decreased tCr (tCr is

more commonly used a reference metabolite) and reflective of increased astrogliosis in SPMS, causing IPS dysfunction.

There was a correlation between tNAA in NAWM and SDMT. However, this association was not significant after adjusting for covariates in the regression analysis. Whilst both PASAT3 and SDMT measure IPS, PASAT3 involves verbal work memory compared to SDMT which involves visuo-spatial memory and this may explain the association between neuroaxonal integrity in NAWM and PASAT3 but not SDMT.[38] A separate study comparing PASAT to SDMT using fMRI found that PASAT activated more frontal areas and the left inferior parietal lobe, suggesting that PASAT required more working memory capacity and executive function to execute compared to SDMT.[39] However, studies in RRMS suggest strong correlation between PASAT3 and SDMT and with similar sensitivity and specificity between the two tests, so our lack of association with SDMT should be interpreted with caution.[40]

A previous study of choline levels in cortical GM did not show any difference between primary progressive MS and healthy controls; nor was there any association with clinical disability measures.[14] Our results suggest an association between GM tCho (a surrogate marker of membrane turnover) and IPS - as GM tCho levels increase, IPS performance (reflected by PASAT3 scores) decreases (see Tables 3 and 4, figure 3). Phosphatidylcholine is one of the major choline containing compounds contained within cell membranes and there is evidence that this along with its precursor molecule phosphocholine and breakdown product glycerophosphocholine are measured by the tCho peak in ^{1}H-MRS.[41] Along with this, the consistent finding of increased tCho (tCho/tCr) in active MS lesions suggests that tCho is a marker of membrane turnover.[9] Recent findings have demonstrated that meningeal lymphoid follicles release pro-inflammatory molecules that can lead to cortical GM inflammation in SPMS and our findings may support this with a surrogate marker of increased membrane turnover in cortical GM being associated with IPS.[42,43] However, this should be interpreted

with caution because tCho was not associated with other measures of clinical disability. Our findings may have differed from the previous study as Sastre-Garriga et al examined a cohort of 41 participants with early PPMS and lower disability (median EDSS 4.5, mean disease duration 3.31 years).[14]

Previous studies exploring the relationship between brain neurometabolites and measures of IPS in PMS did not report the correlation coefficients making it difficult to place our results in the context of previously reported results.[14,16,18,44] Though, examining the associations in these studies between metabolites and other clinical disability measures e.g. EDSS would suggest that the size of our correlations (see table 3) are not out of keeping with previously reported work.[14,16]

Although there were statistically significant correlations in GM between mIns and PASAT3 scores, mIns/tCr and SDMT scores and mIns/tCr and T25FW (see table 3), these were not significant in the regression analysis adjusting for other covariates and is generally in keeping with previous studies that have examined associations between cortical GM metabolites and clinical disability measures such as EDSS and the Brief repeatable battery in SPMS cohorts.[13,45–47] A single study found tNAA was decreased in cortical GM compared to controls and tNAA in cortical GM showed a moderate association with EDSS and 9HPT but this was in early PPMS.[14] When calculating GM metabolites, several studies used careful placement of the VOI using single voxel spectroscopy;[13,46] whilst the remaining studies used CSI followed by automated or manual tissue segmentation.[45,47] These differing techniques reflect the technical challenges in GM metabolite calculation where the partial volume effect can result in contamination of the VOI or leaving insufficient pure GM voxels. The other issue is that previous studies have reported clinical measures that reflect general disability or composite scores e.g. EDSS and Brief repeatable battery, making it difficult to determine associations with specific functional measures such as upper limb function or ambulation. We attempted to

address these issues using CSI followed by segmentation; then using a regression method to calculate GM metabolite values, and then examining these associations with specific functional measures of clinical disability.

Measures of ambulation and general disability:

Whilst there were several associations seen between metabolites and T25W and EDSS (see table 3), after multiple regression analysis we did not find any significant associations between the T25FW performance or the EDSS and any neurometabolite levels. Previous studies examining SPMS cohorts have shown relationships between total choline and EDSS in NAWM; and tNAA/Cr in cortical GM and EDSS in a cohort of PPMS (n=15).[16] The lack of association between metabolites and EDSS could be due to the limited distribution of scores in the cohort where 106/148 had an EDSS score of 6.0-6.5; compounded by the ceiling effect and non-linear characteristics of the EDSS.[35] EDSS particularly between 4.0-6.5 is defined by ambulatory distance and there is a moderate to strong correlation between the T25FW and EDSS.[48] Or there is no relationship to be found.

There were statistically significant correlations between GM Glx (Glx/tCr) and EDSS; and with T25TW (see table 3) but after multiple regression analysis, we did not find any significant relationships between Glx or Glx/tCr and measures of clinical disability. Glutaminergic excitotoxicity has been shown to be involved in the pathogenesis of progressive MS.[4] When glutamate values were measured separately to glutamine using a TE averaged MRSI technique, glutamate was associated with a decline in neuroaxonal integrity in a mixed cohort of MS (number of PMS = 31/343).[49] We measured Glx (glutamine + glutamate) due to the difficulty in resolving glutamate from its precursor glutamine at 3T. It also seems unlikely that we can measure glutaminergic excitotoxicity directly as the majority of Glx signal arises from the intracellular compartment and thereby is more likely to reflect neuro-axonal integrity.

Methodological and analytical considerations

The demographics and characteristics (n=119) of the cohort analysed in this study were consistent with those reported from the main MS-SMART study (n=445).[20] The placement of the CSI grid above the ventricles was designed to limit the effect of ventricles from which there is no metabolite signal, decrease partial volume effect and ensure consistency of shimming across the CSI slice. The predilection of WML to periventricular regions led to voxels containing predominantly WM and GM and meant we were unable to obtain metabolite values from WML. When determining metabolite values for NAWM and GM, we attempted to limit contamination by WML and CSF by excluding voxels that contained 1% WML and 15% CSF. These parameters kept the best balance between the loss of CSI voxels whilst minimising WML and CSF contamination of voxels used in NAWM and GM calculations. During the calculation of metabolite values in NAWM and GM, 29 participants were excluded as metabolite outliers. No formal statistical comparison was undertaken but the characteristics of these 29 participants were generally similar to those in the cohort that underwent analysis (n=119), except to note the median T2 lesion volume was higher in the excluded group (15.6mL v 9.7mL). While we outline a rationale for dealing with multiple comparisons, some caution is advised during interpretation of the associations due to the multiple comparisons being undertaken. Owing to technical difficulties in GM lesions detection, GM lesions were not segmented and this should be acknowledged when interpreting metabolite values and results pertaining to GM.

The neurometabolite ratios to tCr need careful interpretation as tCr is also affected by neuropathology.[11] There are however benefits to using tCr as a stable reference as it removes the need to adjust for head coil loading, T1 and T2 effects.[9] Estimated concentrations are reported using water reference for scaling which can be used to produce absolute concentrations; this in turn carries assumptions within LCModel about the water content of tissue, which is based on reports from normal healthy volunteers,[50] given this assumption and

the fact that at TR=2000ms T1 effects may still be present, we chose to present an estimated (rather than absolute) concentration in institutional units. The use of estimated concentrations allows for the fact that errors between the LCmodel "absolute" concentrations and the true metabolite concentrations may be present.

In summary, after multiple regression analysis tNAA, tNAA/tCr and mIns/tCr in NAWM and tCho in GM are associated with clinical disability in upper limb function and information processing speed. These metabolites are therefore of interest as surrogate markers of brain injury in SPMS.

References

1. Ontaneda D, Thompson AJ, Fox RJ, et al. Series Multiple sclerosis 3 Progressive multiple sclerosis: prospects for disease therapy, repair, and restoration of function. *Lancet* 2017;389:1357–66.

2. Mahad DH, Trapp BD, Lassmann H. Pathological mechanisms in progressive multiple sclerosis. *Lancet Neurol* 2015;14:183–93.

3. Paling D, Golay X, Wheeler-Kingshott C, et al. Energy failure in multiple sclerosis and its investigation using MR techniques. *J Neurol* 2011;258:2113–27.

4. Macrez R, Stys PK, Vivien D, et al. Mechanisms of glutamate toxicity in multiple sclerosis: biomarker and therapeutic opportunities. *Lancet Neurol* 2016;15:1089–102.

5. Brown JWL, Chard DT. The role of MRI in the evaluation of secondary progressive

multiple sclerosis. *Expert Rev Neurother* 2016;16:157–71.

6. Ciccarelli O, Toosy AT, De Stefano N, et al. Assessing neuronal metabolism in vivo by modeling imaging measures. *J Neurosci* 2010;30:15030–3.

7. Rovira À, Alonso J. 1H Magnetic Resonance Spectroscopy in Multiple Sclerosis and Related Disorders. *Neuroimaging Clin N Am* 2013;23:459–74.

8. Bitsch A, Bruhn H, Vougioukas V, et al. Inflammatory CNS Demyelination: Histopathologic Correlation with In Vivo Quantitative Proton MR Spectroscopy. *Am J Neuroradiol* 1999;20:1619–27.

9. Swanberg KM, Landheer K, Pitt D, et al. Quantifying the Metabolic Signature of Multiple Sclerosis by in vivo Proton Magnetic Resonance Spectroscopy: Current Challenges and Future Outlook in the Translation From Proton Signal to Diagnostic Biomarker. *Front Neurol* 2019;10.

10. Narayana PA, Wolinsky JS, Rao SB, et al. Multicentre proton magnetic resonance spectroscopy imaging of primary progressive multiple sclerosis. *Mult Scler* 2004;10 Suppl 1:S73-8.

11. Vrenken H, Barkhof F, Uitdehaag BMJ, et al. MR spectroscopic evidence for glial increase but not for neuro-axonal damage in MS normal-appearing white matter. *Magn Reson Med* 2005;53:256–66.

12. Rovaris M, Gallo A, Falini A, et al. Axonal Injury and Overall Tissue Loss Are Not Related in Primary Progressive Multiple Sclerosis. *Arch Neurol* 2005;62:898–902.

13. Geurts JJG, Reuling IEW, Vrenken H, et al. MR spectroscopic evidence for thalamic and hippocampal, but not cortical, damage in multiple sclerosis. *Magn Reson Med* 2006;55:478–83.

14. Sastre-Garriga J, Ingle GT, Chard DT, et al. Metabolite Changes in Normal-Appearing Gray and White Matter Are Linked With Disability in Early Primary Progressive Multiple Sclerosis. *Arch Neurol* 2005;62:569.

15. Hannoun S, Bagory M, Durand-Dubief F, et al. Correlation of Diffusion and Metabolic Alterations in Different Clinical Forms of Multiple Sclerosis. Najbauer J, ed. *PLoS One* 2012;7:e32525.

16. Obert D, Helms G, Sättler MB, et al. Brain Metabolite Changes in Patients with Relapsing-Remitting and Secondary Progressive Multiple Sclerosis: A Two-Year Follow-Up Study. *PLoS One* 2016;11:e0162583.

17. Foong J, Rozewicz L, Davie CA, et al. Correlates of Executive Function in Multiple Sclerosis: *J Neuropsychiatry Clin Neurosci* 1999;11:45–50.

18. Cox D, Pelletier D, Genain C, et al. The unique impact of changes in normal appearing brain tissue on cognitive dysfunction in secondary progressive multiple sclerosis patients. *Mult Scler J* 2004;10:626–9.

19. Connick P, Angelis F De, Parker RA, et al. Multiple Sclerosis-Secondary Progressive Multi-Arm Randomisation Trial (MS-SMART): a multiarm phase IIb randomised, double-blind, placebo-controlled clinical trial comparing the efficacy of three neuroprotective drugs in secondary progressive multiple sclerosis. *BMJ Open* 2018;8:e021944.

20. Chataway J, De Angelis F, Connick P, et al. Efficacy of three neuroprotective drugs in secondary progressive multiple sclerosis (MS-SMART): a phase 2b, multiarm, double-blind, randomised placebo-controlled trial. *Lancet Neurol* 2020;19:214–25.

21. Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability

status scale (EDSS). *Neurology* 1983;33:1444–52.

22. Cutter GR, Baier ML, Rudick RA, et al. Development of a multiple sclerosis functional composite as a clinical trial outcome measure. *Brain* 1999;122:871–82.

23. Benedict RH, DeLuca J, Phillips G, et al. Validity of the Symbol Digit Modalities Test as a cognition performance outcome measure for multiple sclerosis. *Mult Scler* 2017;23:721–33.

24. Brink HF, Buschmann MD, Rosen BR. NMR chemical shift imaging. *Comput Med Imaging Graph* 1989;13:93–104.

25. Shen J, Rothman DL, Hetherington HP, et al. Linear projection method for automatic slice shimming. *Magn Reson Med* 1999;42:1082–8.

26. Modat M, Ridgway GR, Taylor ZA, et al. Fast free-form deformation using graphics processing units. *Comput Methods Programs Biomed* 2010;98:278–84.

27. Prados F, Cardoso MJ, Kanber B, et al. A multi-time-point modality-agnostic patch-based method for lesion filling in multiple sclerosis. *Neuroimage* 2016;139:376–84.

28. Cardoso MJ, Modat M, Wolz R, et al. Geodesic Information Flows: Spatially-Variant Graphs and Their Application to Segmentation and Fusion. *IEEE Trans Med Imaging* 2015;34:1976–88.

29. Smith SM, Zhang Y, Jenkinson M, et al. Accurate, Robust, and Automated Longitudinal and Cross-Sectional Brain Change Analysis. *Neuroimage* 2002;17:479–89.

30. Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn Reson Med* 1993;30:672–9.

31. Ernst T, Kreis R, Ross BD. Absolute Quantitation of Water and Metabolites in the Human Brain. I. Compartments and Water. *J Magn Reson Ser B* 1993;102:1–8.

32. Solana E, Martinez-Heras E, Martinez-Lapiscina EH, et al. Magnetic resonance markers of tissue damage related to connectivity disruption in multiple sclerosis. *NeuroImage Clin* 2018;20:161–8.

33. R Core Team. R: A language and environment for statistical computing. 2017. [Epub ahead of print].

34. Patel CJ, Ioannidis JPA. Placing epidemiological results in the context of multiplicity and typical correlations of exposures. *J Epidemiol Community Heal* 2014;68:1096–100.

35. Giovannoni G, Cutter G, Pia-Sormani M, et al. Is multiple sclerosis a length-dependent central axonopathy? The case for therapeutic lag and the asynchronous progressive MS hypotheses. *Mult Scler Relat Disord* 2017;12:70–8.

36. De Stefano N, Narayanan S, Francis GS, et al. Evidence of Axonal Damage in the Early Stages of Multiple Sclerosis and Its Relevance to Disability. *Arch Neurol* 2001;58:65–70.

37. Penny S, Khaleeli Z, Cipolotti L, et al. Early imaging predicts later cognitive impairment in primary progressive multiple sclerosis. *Neurology* 2010;74:545–52.

38. Sonder JM, Burggraaff J, Knol DL, et al. Comparing long-term results of PASAT and SDMT scores in relation to neuropsychological testing in multiple sclerosis. *Mult Scler J* 2014;20:481–8.

39. Forn C, Belenguer A, Belloch V, et al. Anatomical and functional differences between the paced auditory serial addition test and the symbol digit modalities test. *J Clin Exp*

Neuropsychol 2011;33:42–50.

40. López-Góngora M, Querol L, Escartín A. A one-year follow-up study of the Symbol Digit Modalities Test (SDMT) and the Paced Auditory Serial Addition Test (PASAT) in relapsing-remitting multiple sclerosis: An appraisal of comparative longitudinal sensitivity. *BMC Neurol* 2015;15:40.

41. Miller BL. A review of chemical issues in1H NMR spectroscopy:N-acetyl-l-aspartate, creatine and choline. *NMR Biomed* 1991;4:47–52.

42. James RE, Schalks R, Browne E, et al. Persistent elevation of intrathecal pro-inflammatory cytokines leads to multiple sclerosis-like cortical demyelination and neurodegeneration. *Acta Neuropathol Commun* 2020;8.

43. Magliozzi R, Howell O, Vora A, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* 2007;130:1089–104.

44. MacMillan E, Tam R, Zhao Y, et al. Progressive multiple sclerosis exhibits decreasing glutamate and glutamine over two years. *Mult Scler J* 2016;22:112–6.

45. Caramanos Z, DiMaio S, Narayanan S, et al. 1H-MRSI evidence for cortical gray matter pathology that is independent of cerebral white matter lesion load in patients with secondary progressive multiple sclerosis. *J Neurol Sci* 2009;282:72–9.

46. Sarchielli P, Presciutti O, Tarducci R, et al. Localized 1 H magnetic resonance spectroscopy in mainly cortical gray matter of patients with multiple sclerosis. *J Neurol* 2002;249:902–10.

47. Adalsteinsson E, Langer-Gould A, Homer RJ, et al. Gray Matter n-Acetyl Aspartate Deficits in Secondary Progressive but Not Relapsing-Remitting Multiple Sclerosis. *Am*

J Neuroradiol 2003;24.

48. Motl RW, Cohen JA, Benedict R, et al. Validity of the timed 25-foot walk as an ambulatory performance outcome measure for multiple sclerosis. *Mult Scler* 2017;23:704–10.

49. Azevedo CJ, Kornak J, Chu P, et al. In vivo evidence of glutamate toxicity in multiple sclerosis. *Ann Neurol* 2014;76:269–78.

50. Ernst T, Kreis R, Ross BD. Absolute Quantitation of Water and Metabolites in the Human Brain. I. Compartments and Water. *J Magn Reson Ser B* https://doi.org/10.1006/jmrb.1993.1055.

Table 1: Baseline demographics and characteristics (n=119)

Clinical Variable	**Mean (standard deviation)**
Age (years)	54.2 (6.7)
EDSS^{+}	6.0 (4.0-6.5)
Normalised brain volume (mL)	1418 (87.3)
Nine-hole peg test (sec^{-1})*	0.035 (0.01)
SPMS duration (years)	22.3 (9.2)
PASAT3 Score (out of 60)	43.0 (11.5)
SDMT Score (out of 110)	46.9 (11.0)
T2 lesion volume^{+} (mL)	9.7 (0.3-34.6)
T25FW (sec)	10.6 (4.3-180.0)

$^{+}$ = median (range), EDSS = Expanded disability status scale, PASAT3 = Paced auditory serial addition task, SDMT = symbol digit modalities test, SPMS = Secondary progressive multiple sclerosis, T25FW = Timed 25-foot walk test

*Nine-hole peg test calculated by taking the reciprocal of the average of two trials for each arm and taking the mean

Table 2 (supplementary appendix):

Summary statistics for neurometabolite estimated concentrations (in institutional units) and ratios to total creatine (n = 119)

Neurometabolite	Mean	SD	Median	Max	Min
NAWM tNAA	8.09	1.04	8.05	10.56	5.62
NAWM tNAA/tCr	1.34	0.14	1.34	1.82	1.08
NAWM mIns	4.72	0.91	4.68	7.03	2.75
NAWM mIns/tCr	0.79	0.13	0.78	1.14	0.52
NAWM Glx	10.01	1.62	9.96	14.23	6.28
NAWM Glx/tCr	1.68	0.23	1.69	2.75	1.07
NAWM tCho	1.46	0.26	1.44	2.19	0.70
NAWM tCho/tCr	0.24	0.03	0.24	0.32	0.17
GM tNAA	9.49	1.96	9.73	13.85	4.21
GM tNAA/tCr	1.36	0.23	1.35	1.88	0.75
GM mIns	5.11	1.41	5.05	8.68	1.60
GM mIns/tCr	0.73	0.21	0.72	1.43	0.19
GM Glx	12.22	3.44	11.91	20.02	5.54
GM Glx/tCr	1.75	0.45	1.77	3.05	0.79
GM tCho	1.60	0.39	1.63	2.34	0.37
GM tCho/tCr	0.23	0.06	0.23	0.39	0.02

Glx = glutamate and glutamine, GM = grey matter, mIns = myoinositol, NAWM = normal appearing white matter, SD = standard deviation, tCho = glycerophosphocholine and phosphocholine, tCr = creatine and phosphocreatine, tNAA = N-acetyl aspartate and N-acetyl aspartyl glutamate

Table 3. Spearman Rank correlations between neurometabolites (Estimated concentrations and ratios) and disability measures (n = 119).

	EDSS			Timed 25 foot walk			Nine-hole peg test			PASAT3 scores			SDMT Scores		
	Rho	95% CI		Rho	95% CI		Rho	95% CI		Rho	95% CI		Rho	95% CI	
NAWM tNAA	0.10	-0.08	0.28	0.07	-0.11	0.25	**0.22***	**0.05**	**0.39**	**0.21***	**0.03**	**0.38**	**0.20***	**0.02**	**0.37**
NAWM tNAA/tCr	0.02	-0.17	0.19	-0.05	-0.23	0.13	**0.23***	**0.06**	**0.40**	**0.19***	**0.01**	**0.36**	0.14	-0.05	0.31
NAWM mIns	-0.11	-0.29	0.07	-0.06	-0.24	0.12	-0.01	-0.19	0.17	-0.15	-0.32	0.03	0.00	-0.18	0.18
NAWM mIns/tCr	**-0.26****	**-0.42**	**-0.09**	**-0.20***	**-0.37**	**-0.03**	-0.03	-0.21	0.15	**-0.23***	**-0.39**	**-0.05**	-0.08	-0.26	0.10
NAWM Glx	0.08	-0.10	0.26	0.10	-0.08	0.28	0.00	-0.18	0.18	0.00	-0.18	0.18	**0.19***	**0.01**	**0.36**
NAWM Glx/tCr	0.03	-0.15	0.21	0.07	-0.11	0.25	-0.12	-0.29	0.07	-0.12	-0.30	0.06	0.04	-0.15	0.22
NAWM tCho	-0.10	-0.27	0.08	0.00	-0.18	0.18	0.09	-0.09	0.26	0.17	-0.01	0.34	0.17	-0.01	0.34
NAWM tCho/tCr	-0.22	-0.39	-0.04	-0.12	-0.30	0.06	0.05	-0.14	0.22	0.12	-0.06	0.29	0.07	-0.12	0.24
GM tNAA	-0.16	-0.33	0.02	-0.14	-0.31	0.04	0.05	-0.13	0.22	0.02	-0.16	0.20	0.08	-0.10	0.26
GM tNAA/tCr	-0.05	-0.23	0.13	-0.01	-0.19	0.17	0.08	-0.11	0.25	0.05	-0.13	0.23	0.06	-0.12	0.24
GM mIns	0.10	-0.08	0.28	0.18	0.00	0.34	-0.14	-0.32	0.04	**-0.20***	**-0.37**	**-0.02**	-0.10	-0.27	0.09
GM mIns/tCr	0.18	0.00	0.35	**0.28****	**0.10**	**0.44**	-0.15	-0.32	0.03	-0.14	-0.32	0.04	**-0.18***	**-0.35**	**0.00**
GM Glx	**-0.28****	**-0.44**	**-0.10**	**-0.30*****	**-0.45**	**-0.13**	0.04	-0.14	0.22	0.14	-0.04	0.31	0.03	-0.15	0.21
GM Glx/tCr	**-0.22***	**-0.39**	**-0.04**	**-0.26*****	**-0.42**	**-0.08**	0.06	-0.12	0.24	0.19	0.01	0.36	-0.02	-0.20	0.17
GM tCho	0.11	-0.07	0.28	0.11	-0.07	0.29	-0.05	-0.23	0.13	**-0.24****	**-0.40**	**-0.06**	-0.08	-0.25	0.11
GM tCho/tCr	0.16	-0.02	0.33	0.19	0.01	0.36	0.00	-0.18	0.18	**-0.21***	**-0.37**	**-0.03**	-0.09	-0.27	0.09

Statistically significant correlation coefficients are highlighted in bold text. $p < .001$ ***, $p < .01$ **, $p < .05$ *

EDSS = Expanded disability status scale, GM = grey matter, Glx = glutamate and glutamine, mIns = myoinositol, NAWM = normal appearing white matter, PASAT3 = Paced auditory serial addition task, SDMT = symbol digit modalities test, tCho = glycerophosphocholine and phosphocholine, tCr = creatine and phosphocreatine, tNAA = N-acetyl aspartate and N-acetyl aspartyl glutamate

Table 4: Results from multiple regression analysis examining associations between neurometabolites and clinical disability measures

Nine-hole peg test* (n = 118)**			
Predictors	*std. Beta*	*std 95% CI*	*p*
NAWM tNAA/tCr	0.19	0.01 – 0.36	0.04
Paced auditory serial addition task (n = 119)			
Predictors	*std. Beta*	*std 95% CI*	*p*
NAWM tNAA	0.17	0.01 – 0.34	0.04
Gender*	-0.23	-0.42 to -0.05	0.01
T2 lesion volume	-0.47	-0.64 to -0.30	<0.001
Paced auditory serial addition task (n = 118)**			
Predictors	*std. Beta*	*std 95% CI*	*p*
NAWM tNAA/tCr	0.19	0.03 – 0.35	0.02
Gender*	-0.26	-0.44 to -0.08	0.006
T2 lesion volume	-0.46	-0.63 to -0.29	<0.001
Paced auditory serial addition task (n = 119)			
Predictors	*std. Beta*	*std 95% CI*	*p*
NAWM mIns/tCr	-0.22	-0.39 to -0.06	0.007
Gender*	-0.25	-0.43 to -0.07	0.008
T2 lesion volume	-0.47	-0.64 to -0.30	<0.001

Paced Auditory Serial Addition Task (n = 119)			
Predictors	*std. Beta*	*std 95% CI*	*p*
GM tCho	-0.17	-0.33 – -0.01	0.04
T2 lesion volume	-0.48	-0.65 – -0.31	<0.001

*Male is reference category. ** 118 participants in this cohort as 1 case removed due to a highly leveraged point ***Nine-hole peg test calculated by taking the reciprocal of the average of two trials for each arm and taking the mean

CI = confidence interval, GM = grey matter, mIns = myoinositol, NAWM = normal appearing white matter, SE = standard error, std = standardised, tCho = glycerophosphocholine and phosphocholine, tCr = creatine and phosphocreatine, tNAA = N-acetyl aspartate and N-acetyl aspartyl glutamate.

Covariates in model include: age, gender, duration from onset, occurrence of relapse in 2 years preceding randomisation, T2 lesion volume and normalised brain volume. Table highlights only the predictor variables that were significant from the multiple regression models

Figure 1. Proton magnetic resonance spectroscopy slice placement and mask

The top panel demonstrates slice placement in the sagittal, coronal and axial planes. The lower panel shows an example of the chemical shift mask for normal appearing white matter and grey matter.

Figure 2. Example spectra and chemical shift grid

Figure 2 shows A) Position where representative spectra come from including a lesion, B) accepted spectra in red and lesion in black, C) spectra from rejected voxel, position shown in green in A.

Figure 3. Associations between neurometabolites and clinical disability measures

9HPT = nine-hole peg test, IU = Institutional units, mIns = myoinositol, NAWM = normal appearing white matter, PASAT3 = Paced auditory serial addition task, tCr = creatine and phosphocreatine, tNAA = N-acetyl aspartate and N-acetyl aspartyl glutamate

The scatterplots with the line of best fit and 95% confidence intervals are shown for the associations in normal appearing white matter between A. tNAA/tCr and nine-hole peg test [Rho=0.23, 95% CI [0.06-0.40], B. tNAA and Paced auditory serial addition task, [Rho=0.21, 95% CI [0.03-0.38] C. tNAA/tCr and Paced auditory serial addition task [Rho=0.19, 95% CI [0.01-0.36] D. mIns/tCr and Paced auditory serial addition task [Rho=-0.23, 95% CI [-0.39 to -0.05] and in grey matter between E. tCho and Paced auditory serial addition task [Rho=-0.24, 95% CI [-0.40 to -0.06].